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UTILITY PATENT APPLICATION TRANSMITTAL
(Small Entity)

(Only for new nonprovisional applications under 37 CFR 1.53(b))

Docket No. 0168.00079
Total Pages in this Submission

TO THE ASSISTANT COMMISSIONER FOR PATENTS
Box Patent Application
Washington, D.C. 20231

Transmitted herewith for filing under 35 U.S.C. 111(a) and 37 C.F.R. 1.53(b) is a new utility patent application for an invention entitled:

GENE IDENTIFICATION METHOD

and invented by:

Louis Paul Deiss et al.

If a CONTINUATION APPLICATION, *check appropriate box and supply the requisite information:*

☐ **Continuation** ☐ **Divisional** ☒ **Continuation-in-part (CIP)** of prior application No.: **09/294,782**

Which is a:

☐ **Continuation** ☐ **Divisional** ☐ **Continuation-in-part (CIP)** of prior application No.: _____

Which is a:

☐ **Continuation** ☐ **Divisional** ☐ **Continuation-in-part (CIP)** of prior application No.: _____

Enclosed are:

Application Elements

1. ☒ Filing fee as calculated and transmitted as described below
2. ☒ Specification having **61** pages and including the following:
   - a. ☒ Descriptive Title of the Invention
   - b. ☒ Cross References to Related Applications *(if applicable)*
   - c. ☐ Statement Regarding Federally-sponsored Research/Development *(if applicable)*
   - d. ☐ Reference to Microfiche Appendix *(if applicable)*
   - e. ☒ Background of the Invention
   - f. ☒ Brief Summary of the Invention
   - g. ☒ Brief Description of the Drawings *(if drawings filed)*
   - h. ☒ Detailed Description
   - i. ☒ Claim(s) as Classified Below
   - j. ☒ Abstract of the Disclosure

EL405597895US
2-7-00

Constance McLean

UTILITY PATENT APPLICATION TRANSMITTAL
(Small Entity)

(Only for new nonprovisional applications under 37 CFR 1.53(b))

Docket No. 0168.00079
Total Pages in this Submission

Application Elements (Continued)

3. ☒ Drawing(s) *(when necessary as prescribed by 35 USC 113)*
   a. ☐ Formal b. ☒ Informal Number of Sheets 13

4. ☒ Oath or Declaration
   a. ☐ Newly executed *(original or copy)* ☒ Unexecuted
   b. ☐ Copy from a prior application (37 CFR 1.63(d)) *(for continuation/divisional application only)*
   c. ☒ With Power of Attorney ☐ Without Power of Attorney
   d. ☐ *DELETION OF INVENTOR(S)*
   Signed statement attached deleting inventor(s) named in the prior application, see 37 C.F.R. 1.63(d)(2) and 1.33(b).

5. ☐ Incorporation By Reference *(usable if Box 4b is checked)*
The entire disclosure of the prior application, from which a copy of the oath or declaration is supplied under Box 4b, is considered as being part of the disclosure of the accompanying application and is hereby incorporated by reference therein.

6. ☐ Computer Program in Microfiche

7. ☐ Genetic Sequence Submission *(if applicable, all must be included)*
   a. ☐ Paper Copy
   b. ☐ Computer Readable Copy
   c. ☐ Statement Verifying Identical Paper and Computer Readable Copy

Accompanying Application Parts

8. ☐ Assignment Papers *(cover sheet & documents)*

9. ☐ 37 CFR 3.73(b) Statement *(when there is an assignee)*

10. ☐ English Translation Document *(if applicable)*

11. ☐ Information Disclosure Statement/PTO-1449 ☐ Copies of IDS Citations

12. ☒ Preliminary Amendment

13. ☒ Acknowledgment postcard

14. ☒ Certificate of Mailing
   ☐ First Class ☒ Express Mail *(Specify Label No.):* EL 405 597 895 US

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Accompanying Application Parts (Continued)

15. ☐ Certified Copy of Priority Document(s) *(if foreign priority is claimed)*

16. ☒ Small Entity Statement(s) - Specify Number of Statements Submitted: 1

17. ☐ Additional Enclosures *(please identify below):*

Fee Calculation and Transmittal

CLAIMS AS FILED

For	#Filed	#Allowed	#Extra		Rate	Fee
Total Claims	9	- 20 =	0	x	$9.00	$0.00
Indep. Claims	2	- 3 =	0	x	$39.00	$0.00
Multiple Dependent Claims (check if applicable) ☐						$0.00
					BASIC FEE	$345.00
OTHER FEE *(specify purpose)*						$0.00
					TOTAL FILING FEE	$345.00

☒ A check in the amount of **$345.00** to cover the filing fee is enclosed.

☒ The Commissioner is hereby authorized to charge and credit Deposit Account No. **11-1449** as described below. A duplicate copy of this sheet is enclosed.

- ☐ Charge the amount of as filing fee.
- ☒ Credit any overpayment.
- ☒ Charge any additional filing fees required under 37 C.F.R. 1.16 and 1.17.
- ☐ Charge the issue fee set in 37 C.F.R. 1.18 at the mailing of the Notice of Allowance, pursuant to 37 C.F.R. 1.311(b).

Dated: **February 7, 2000**

Signature

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cc:

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:

LOUIS PAUL DEISS ET AL.

Serial No.: Unknown

Filed: Herewith

For: GENE IDENTIFICATION METHOD

Our File No.: 0168.00079

PRELIMINARY AMENDMENT

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

Please preliminarily amend the above-captioned patent application prior to examination:

IN THE SPECIFICATION:

Page 1, after the title, please insert the following subparagraph:

-- **CROSS-REFERENCE TO RELATED APPLICATIONS**

This application is a continuation-in-part of United States Patent Application Serial No. 09/284,782, filed July 6, 1999, which is a National Phase of International Serial No. PCT/US97/20989, filed November 12, 1997. --

Page 4, line 5, after "library" please delete the period and insert the following paragraphs:

--Figure 3 is a schematic representation of the AHM method of the present invention;

Figures 4 A-C show the effect of the AHM method on cell proliferation; A. shows how anti-sense bFGF sensitizes HeLa cells to Fas induced PCD; B. shows the levels of expression of bFGF; C. shows the quantitation of the levels bFGF forms;

Figures 5 A-D show the effect of the AHM method on cell proliferation; A. shows how anti-sense Nrf2 sensitizes HeLa cells to Fas induced PCD; B. shows the levels of expression of Nrf2; C. shows how Dicumarol sensitizes HeLa cells to Fas induced PCD; D. shows how N-acetyl cysteine protects HeLa cells from Fas induced PCD; and

Figures 6 A-C show the effect of the AHM method; A. shows "Function Profiling" generated by cDNA microarray analysis; B. shows the distribution of the differential abundance of cDNAs contained in the microarray; C. shows "Sensitizing" cDNAs candidates identified by microarray analysis. --

Page 17, line 19, after "1990.], please insert the following new paragraphs:

-- Also disclosed by the present invention is a method for the identification of genes that encode for inhibitors of cell death. This method is commonly known as the achilles heel method (AHM). This method involves introducing an antisense library into a vector such as an episomal vector (Deiss and Kimchi, 1991) enters target cells to generate a pool of cells with each cell expressing a different antisense fragment. This pool of cells will be known as Pool 1. Second, the transfactents are treated with a sub-optimal dose of an

inducer and the surviving cells are collected. The surviving cells are known as Pool 2. The cells containing inactivation events that sensitize the cells to death are preferentially lost from Pool 2, and so are the antisense CDNA inserts that confer the sensitization. These CDNA inserts are recovered by subtracting the CDNA inserts containing in Pool 2 from those in Pool 1. The products of the subtraction are cloned in the episomal expression vector and individually transfected into target cells in order to confirm their ability to render the cells more sensitive to the killing inducer.

Following the subtraction of Pool 2 cDNAs from Pool 1 cDNAs, the potentially sensitizing cDNAs are cloned in an anti-sense orientation in an episomal expression vector. The anti-sense cDNA containing episomes are individually transfected into target cells in order to confirm their ability to render the cells more sensitive to the killing inducer. Alternatively, Pool 1 and Pool 2 cDNAs are labeled and used as probes for hybridization of cDNA microarray filter. Computer analysis identifies the cDNAs depleted from Pool 2. In both cases "function profiling" is being employed to identify signal pathway inhibitors. Recently, similar "function profiling" methods have been described for genetic analysis of S.cerevisae by .. et al (Pat Brown and Ron Davis). These methods are well suited to yeast since they require prior knowledge of gene sequence and the ability to generate haploid cells. By contrast, AHM does not require a priori knowledge of any gene sequence or haploid cells. Thus, AHM is a powerful genetic tool for "function profiling" in mammalian cells. Moreover, AHM can be easily scaled up to generate "function profiles" of all expressed human genes.

The AHM and the TKO methods are complimentary and together can be used to identify the positive and negative regulators of any pathway that can be reconstructed into human cells and culture. The AHM method identifies genes whose inactivation by antisense sensitize cells to an inducer. Thus, it

enables dissection of the signalling pathway by identifying inhibitors of cell death. Since these two methods are related, the subtraction analysis is similar to that disclosed above.

There are several uses for the gene that were identified in the AHM screen for inhibitors of Fas induced apoptosis. Since the activation of the fas pathway is relevant to several different pathologies it is useful to have modifiers of Fas induced killing. The activation of the Fas pathway is associated with detrimental effects such as liver damage in fulminant hepatitis or immune mediated tissue destruction. Conversely, proper activation of the Fas pathway is required for elimination of autoreactive T-cells and it has been shown that activation of the Fas pathway mediates some aspects of tumor suppression. Thus it would be clinically useful to be able to enhance or inhibit the Fas pathway depending on the particular situation. The identification of inhibitors of the Fas pathway can be used for both of these purposes. The inhibitors should act to sensitize cell to Fas induced killing. Thus inhibition of Nrf-2 by anti-sense inactivation sensitizes cells to killing induced by Fas. It has been shown that the inhibition of genes regulated by Nrf-2 such as Glutathione-S-transferase and NAD(P)H quinone oxireductase by Dicumarol also sensitizes cells to Fas induced killing. This shows that Dicumarol or drugs that act in a similar manner may also enhance Fas induced killing in other situations. Specifically these agents can enhance the killing of auto-reactive T-cells by Fas and thereby reduce the symptoms of auto-immunity. This can be done during the early stages of the disease since some of the ultimate tissue damage induced in auto-immunity is mediated by activation of the Fas pathway. Similarly, the enhancement of Fas induced killing can be beneficial in mediating some aspects of tumor suppression since it has been demonstrated that some tumor suppression activities are mediated through the activation of the Fas receptor. Thus the finding that Dicumarol *sensitizes* to Fas induced killing can be proposed to be a remedy for auto-immunity.

Similarly the finding that Sulfinpyrazone also sensitizes to Fas induced cell killing also shows that Sulfinpyrazone as well as drugs that act similarly can be remedies for auto-immunity.

Conversely, the inhibition of Fas activated killing can also have clinical benefit under certain circumstances. For example the inhibition of Fas induced killing can protect the liver from acute damage. The inhibition of the Fas pathway by over expression of the gene identified in the AHM Fas screen inhibits Fas induced cell killing. These genes are secreted molecules, thus the addition of the soluble molecule protect cells from damage. Over expression of the inhibitors can also be achieved through a number of other means including the use of gene therapy to transduce the gene into potential target cells and protect those cells.

There are other uses of the AHM method that are directly related to the validation of AHM in identifying inhibitors of the Fas pathway. One application is to identify toxic drug interactions. Performing an AHM screen to identify genes that inhibit toxic side effects of a specific drug (drug X) is one example. It is important that the genes that are identified as inhibitors of drug X induced toxicity be fully functioning. When one of the identified genes is found to be inhibited by another drug (drug Y) then that drug Y sensitizes patients to drug X. This type of analysis permits rapid screening for drug interactions before the drugs were used on patients.

The genes identified in the Fas AHM screen are survival factors since they inhibit killing induced by Fas. As such they can be used to limit chronic or acute pathological situations in which there is excessive cell death. Such applications could include organ failure induced by heart failure, acute liver damage and ischemic stress.

Additionally, the identified targets can then be used as targets to develop inhibitors and those inhibitors can be used as cofactors to activate the identified pathway.

For example, one can identify *genes*, which inhibit killing induced by chemotherapeutics. Inhibition of such genes sensitizes tumors to chemotherapeutics. Such inhibitors have utility in treating cancer patients.

A list of genes were identified in the Fas AHM screen using the gene blot analysis. These genes are identified as inhibitors of Fas induced killing and are shown in Table 3.--

Page 44, line 14, after "selected phenotype." please insert the following paragraphs:

--EXAMPLE 3:

The Achilles Heel Method utilizes functional profiling as diagrammed in Figure 3. The first step consists of introducing an anti-sense expression library (Deiss and Kimchi, 1991) into target cells to generate a pool of cells, each expressing a different anti-sense fragment (Pool 1). Then, the transfectants are treated with a sub-optimal dose of a PCD inducer and the surviving cells are collected (Pool 2). Cells containing inactivation events that sensitize the cells to killing are preferentially lost from Pool 2. Consequently, the anti-sense cDNAs contained in the sensitized cells are depleted from Pool 2. The "sensitizing" cDNA inserts that are present in Pool 1 but depleted from Pool 2 are identified by two methods, subtraction or hybridization to cDNA microarray. Following the subtraction of Pool 2 cDNAs from Pool 1 cDNAs, the potentially sensitizing cDNAs are cloned in an anti-sense orientation in an episomal expression vector. The anti-sense cDNA containing episomes are

individually transfected into target cells in order to confirm their ability to render the cells more sensitive to the killing inducer. Alternatively, Pool 1 and Pool 2 cDNAs are labeled and used as probes for hybridization of cDNA microarray filter. Computer analysis identifies the cDNAs depleted from Pool 2. In both cases "function profiling" is being employed to identify signal pathway inhibitors. Recently, similar "function profiling" methods have been described for genetic analysis of S.cerevisae by .. et al (Pat Brown and Ron Davis). These methods are well suited to yeast since they require prior knowledge of gene sequence and the ability to generate haploid cells. By contrast, AHM does not require a priori knowledge of any gene sequence or haploid cells. Thus, AHM is a powerful genetic tool for "function profiling" in mammalian cells. Moreover, AHM can be easily scaled up to generate "function profiles" of all expressed human genes.

AHM is used to identify inhibitors of the Fas induced PCD pathway. Fas is a trans-membrane death receptor of the TNF super family. The binding of Fas ligand to Fas results in the cascade of events that lead in most cell types to apoptosis. Fas induced killing is utilized in different physiological processes as follows: (for review see Nagata, Gloldstein etc): elimination of auto-reactive T-cells, tumor induced immune suppression and destruction of virally infected cells, transformed cells and b-cells in cases of Insulin Dependent Diabetes Melitus (IDDM). In addition, activation of the Fas pathway has been suggested to play a role in liver damage, brain damage, arteriosclerosis and tumor suppression. Modulation of the Fas pathway has clinical implications in animal models: inhibition of Fas induced PCD by caspase inhibitors limits liver damage in mice and acceleration of Fas induced killing ameliorate the auto-immune phenotype of gld mice. Thus, identifying regulators of the Fas pathway that can be used as targets for drug development will have great clinical impact.

For the identification of inhibitors of Fas induced cell death, AHM was applied to HeLa cells that were treated with sub-lethal dose of Fas agonistic antibody. The later mimics the binding of Fas ligand to Fas and induces apoptosis. "Function profiling" was performed to identify "sensitizing" cDNA fragments by using subtraction and gene array analysis. cDNA inserts from Pool 2 were subtracted from Pool 1 cDNAs and the recovered cDNAs were further analyzed. Sequencing of 226 fragments revealed 168 unique sequences, of which 53% are novel and 47% correspond to known genes. Six out of seven randomly chosen cDNAs that were individually transfected into HeLa cells conferred increased sensitivity to Fas induced killing cells, ranging between 2.9 to 5.3 fold. These fragments include three novel sequences and three fragments of previously described genes. One of the cDNA inserts is aan anti-sense fragment of human Basic Fibroblast Growth Factor (FGF-2, bFGF) and the other is an anti-sense fragment of the cap-n-collar b-zip transcription factor NF-E2 related factor 2 (NRF2).

bFGF is a potent survival factor that plays a role in development, angiogenisis and in cell migration. Previous reports show that down regulation of bFGF by anti-sense expression or by blocking antibodies result in loss of a transform phenotype, reduced tumor growth and reduced angiogenesis. Five different polypeptides of 34kD, 24kD, 22.5kD, 22kD and 18 kD are translated from the human bFGF gene, initiating at different sites and terminating at the same position. The anti-sense cDNA fragment isolated in the subtraction is 295 nucleotides long and corresponds to nucleotide 890 to 1184 of the bFGF gene. It spans the last 60 nucleotides of the coding region (shared by all bFGF polypeptides) and a portion of the 3' untranslated region.

In order to confirm that anti-sense bFGF confers sensitivity to Fas, pools of cells transfected with control vector (harboring no insert) or with anti-sense bFGF were generated and treated with sub-optimal dose of anti-Fas

antibody. Analysis of two independent pools of transfectants demonstrates that under conditions that results in 59% and 29% killing of the vector transfected cells, anti-sense bFGF transfected cells are 3.7 and 4.4 fold more sensitive to killing (Figure 4A). This significant increase sensitivity of anti-sense bFGF transfected cells was reproducible in six independent pools of transfectants. It is not due to altered growth rate of the anti-sense bFGF transfected cells (compare the number of untreated cells in the control vector transfected pools to the number of untreated cells in the anti-sense bFGF transfected pools) or to a non-specific increase in sensitivity of anti-sense transfected cells to Fas induced killing since anti-sense cDNAs were previously isolated that render transfected cells resistant to Fas induced apoptosis. Complementary to the bioassay experiments, quantitative Southern analysis of Pool 1 and Pool 2 indicates that the abundance of anti-sense bFGF cDNA is reduced by 1.9 fold in Pool 2 of cells surviving sub-lethal dose of anti-Fas antibody, compared to Pool 1.

Western blot analysis of control vector transfected cells as well as anti-sense bFGF transfected cells revealed four polypeptides of 24kD, 22/22.5kD and 18kD, while the 34kD form is not detected by the antibody used (Figure 4B). Quantitative analysis of the relative level of bFGF forms revealed that in the absence of anti-Fas antibody, expression of anti-sense bFGF results in reduction of approximately 25%-30% in the levels of each of the detected forms (Figure 4C). Interestingly, in cells treated with anti-Fas antibody a more significant reduction in the levels the 24kD form and 18kD is observed, 57% and 66% respectively, while the reduction of the levels of the 22/22.5kD is not altered. Selective reduction in the level of some of the bFGF forms by an anti-sense fragment that overlaps the coding region of all the bFGF polypeptides can be due to a network of feedback regulation loops as previously reported for some bFGF forms.

While previous studies has shown that over-expression of the 34kD form protects cells from serum deprivation induced killing and over-expression of the 24kD form protects cells from ionizing radiation, here it is demonstrated that bFGF is an inhibitor of Fas induced apoptosis, as identified by AHM.

The second inhibitor of the Fas pathway that was identified by AHM is the cap-n-collar b-zip transcription factor NF-E2 related factor 2 (NRF2). Nrf2 activates the transcription of phase II detoxifying enzymes such as NAD(P)H quinone oxireductase (NQO1) and Glutathione S-transferase (GST) by direct binding to the Antioxidant Response Element (ARE) in the promoter of these genes. Studies of nNrf2 null mice indicate that Nrf2 is essential for the transcriptional activation of phase II enzymes. NQO1 and GST act in concert with phase I detoxifying enzymes (such as cytochrome p-450 monooxygensase) to mediate the cellular detoxification of xenobiotics. In the absence of Nrf2, this coordinated detoxification is impaired and toxic products from phase I reactions can accumulate. In the AHM screen an anti-sense fragment of Nrf2 corresponding to nucleotide 147 to 970 of the human Nrf2 (gbS74017, Moi et al, 1994, PNAS 21, 9926) was recovered.

Bioassays of two pools of HeLa cells transfected with anti-sense Nrf2 clearly demonstrates that anti-sense Nrf2 render the cells 4.1 and 5.4 fold more sensitive to Fas induced apoptosis (Figure 5A). Again, this increase sensitivity is not a result of impaired growth, since there is only limited alteration in the growth rate of anti-sense Nrf2 transfected cells (Figure 5A). Sensitization by anti-sense Nrf2 was reproducible in seven independent pools of transfectants. Western blot analysis indicated a significant 3.8 fold reduction in the level of Nrf2 protein in the anti-sense Nrf2 transfected cells (Figure 5B).

The role of Nrf2 as an inhibitor of the Fas pathway was further validated by pharmacological agents. It is predicted that treatment of cells with Dicumarol, an inhibitor of GST and NQO1 will sensitize cells to Fas induced apoptosis, since Nrf2 up-regulates the levels of GST and NQO1. As shown in Figure 5C, HeLa cells treated with 100mM Dicumarol are 2.8 fold more sensitive to Fas induced killing compared to cells treated with vehicle (fold sensitization was calculated as described in Figure 4A).

Since down regulation of Nrf2 sensitizes cell to Fas induced apoptosis, it was questioned whether increasing any of the activities induced by Nrf2 will protect HeLa cells from apoptosis. Nrf2 up-regulates GST that conjugates glutathione to the reactive products of phase I detoxification. Increased activity of GST will protect cells from Fas induced apoptosis. GST activity was elevated by treating HeLa cells with the glutathione precursor N-acetyl Cysteine (NAC) that increases the glutathione pool. As shown in Figure 5D, NAC strongly protects HeLa cells from Fas induced apoptosis as previously reported for microglia, neutrophils and T-cells.

Thus, by using AHM Nrf2 was identified as an inhibitor of Fas induced PCD in HeLa cells and this result was validated by genetic and pharmacological approaches. Interestingly, Ohtsubo et al has recently reported that Nrf2 is cleaved by caspase-3, producing a fragment that acts as a dominant negative fashion and is lethal. The mechanism underlying the mode of action of Nrf2 in regulating apoptosis deserves further investigation.

A technically simpler alternative to "function profiling" by subtraction is analysis of cDNA microarray. The relative abundance of cDNAs was measured in Pool 1 and in Pool 2 by radio-labeling each pool and hybridizing each of the probes to a cDNA microarray containing approximately 4,000 different known human genes. Figure 6A is a pseudo colored image of

the microarray filter representing for each cDNA spot the ratio of signal generated by hybridization to Pool 2 probe to that generated by Pool 1 probe. Dark green spots indicate cDNAs absent in Pool 2, representing "sensitizing" anti-sense cDNAs. The corresponding genes are predicted to be survival factors that inhibit Fas induced apoptosis. Dark red spots indicate cDNAs that are enriched in Pool 2. These genes are positive mediators of killing and their inactivation by anti-sense results in resistance to PCD. The abundance of such anti-sense cDNAs is therefore increased in Pool 2 that is comprised of cells that survived Fas induced apoptosis. Most of the spots are of intermediate color indicating only modest changes in abundance in Pool 2 relative to Pool 1. A histogram representation of the results is shown in Figure 6B. As seen, the abundance of the majority of cDNAs is not changed. However, a small number of cDNAs are depleted from Pool 1 by 2 folds or more. A partial list of these genes is presented in Figure 6C. As predicated, these genes include survival factors. For example, the most depleted cDNA (5.6-fold) corresponds to TNF receptor associated factor 6 that relays a strong survival signal via the activation of NFkB and AKT. In addition casein kinase 1 alpha and adenosine A3 receptor have been shown to be survival factors.

In summary, this is a novel powerful tool for identifying signaling inhibitors in human cells. Thus, a large gap in the genetic analyses of mammalian cells has now been filled. AHM can be broadly used to identify inhibitors of any given selectable pathway for the purposes of basic research or clinical applications. Moreover, since it does not require previous knowledge of any sequences, AHM can be employed as a high throughput method of gene discovery and "function profiling" as part of the ongoing effort of deciphering the human genome.

Materials and Methods:

AHM: HeLa cells (10^6 cells/100 mm plate) were transfected with 15 ug of anti-sense cDNA library in pTKO-1 (Deiss and Kimchi 1991) by Superfect reagent (Qiagen). Two days later cells were treated with 200 ug/ml Hygromycin B (Calbiochem-Novabiochem) for two weeks. $2.5X10^6$ Hygromycin^R cells were plated in a 150 mm plate 24 hours prior to treatment with10 ng/ml anti-Fas antibody (clone CH-11, Kamiya Biomedical Company) (Pool 2). Five days post treatment approximately 30-40% of the cells were killed as estimated by microscopic examination. A parallel culture was grown in the absence of anti-Fas antibody (Pool 1). After five days, cells were washed twice with PBS, scraped off the plate and stored as pellets at -80^0. 100 ul of frozen pellet were lysed by addition of 200 ul of solution P1, followed by 200 ul of solution P2 . After the lysate sat on ice for 5 minutes 200 ul of solution P3 was added(Qiagen plasmid purification kit). Following 5 minutes incubation on ice, the lysate was centrifuged for 10 minutes at 15,000xg, the supernatant was mixed with an equal volume of isopropanol and centrifuged at 15,000xg for 10 minutes. The DNA pellet was rinsed with 70% ethanol and resuspended in 100 ul of water. The cDNA inserts were amplified by PCR in a 100 ul reaction containing: 1 ul DNA, 200 uM of dATP, dGTP, dCTP, dTTP; 10 mM Tris-HCl pH9.0; 0.1 % Triton X-100; 1.0mM MgCl; 1 unit Taq DNA polymerase (Gibco BRL) and 500 ng each of primers: prLPD#64 (TGGAGGCCTAGGCTTTTGC) and prLPD#65 (GTAAGGTTCCTTCACAAGGATCC). These primers are derived from the sequences that flank the cDNA insertion site in the pTKO-1 anti-sense expression vector. The primers are designed to restore a HindIII restriction site on the promoter proximal side of the cDNA and a BamHI site on the promoter distal side to conserve the orientation of the cDNA fragments upon their cloning in pTKO-1. The reaction was incubated 94^{0}C for 5 minutes; subjected to 25 cycles of: 94^{0}C for one minute, 58^{0}C for one minute and 72^{0}C for five minutes; followed by 72^{0}C for seven minutes. The PCR products were cleaved by BamHI and HindIII, purified (Wizard PCR Prep Kit, Promega) and used in subtraction (PCR-Select kit, Clontech). The driver for the subtraction

was the product of the PCR reaction derived from the untreated cells (Pool 1) and the tester was derived from treated cells (Pool 2). The following modifications to the manufacturer's instructions were made: 1. The first step was IV F 3, since no cDNA synthesis is required. 2. The blunt ends adapters 1 and 2R were replaced with cohesive ends adapters as follows: Adapter I was replaced by a mixture of primers prLPD#80 (CTAATACGACTCACTATAGGGCTCGAGCGGCCGCCCGGGCAGGTA), prLPD#81 (CTAATACGACTCACTATAGGGCTCGAGCGGCCGCCCGGGCAGGTG), prLPD#83 (AGCTTACCTGCCCGG) and prLPD#84 (GATCCACCTGCCCGG). Adapter 2R was replaced by a mixture of prLPD#82 (CTAATACGACTCACTATAGGGCAGCGTGGTCGCGGCCGAGGTG), prLPD#88 (AGCTTACCTCGGCCG), prLPD#89 (GATCCACCTCGGCCG) and prLPD#90 (CTAATACGACTCACTATAGGGCAGCGTGGTCGCGGCCGAGGTA).

Cohesive end adapters ligate more efficiently to the cDNA and permit the directional cloning of the cDNA inserts. 0.3 ug of the tester was used for adapter ligation. 3. The initial hybridization included 0.9 ug of the driver and 0.03 ug of the adapted ligated tester. The products of the subtraction were cleaved with BamHI and HindIII, purified and cloned into the pTKO-1 between BglII and HindIII sites. Individual clones were sequenced and transfected into HeLa cells.

Transfection and Bioassays: HeLa cells ($2x10^6$ cells/100 mm plate) were plated 20 hours prior to transfection with either 17 ug of either anti-sense expressing vector or control vector harboring no cDNA insert, by calcium phosphate. Forty eight hours post transfection cells were treated with 200 ug/ml Hygromycin B (Calbiochem-Novabiochem) for two weeks. For bioassays, anti-sense transfected cells or control vector transfected cells ($1.6 x10^5$ cells/

well in 6 wells plates) were plated 20-24 hours prior to the treatment with 200 ng/ml anti-Fas antibody (clone CH-11, Kamiya Biomedical Company). The number of viable, trypan blue (Gibco/BRL) excluding cells that remained attached to the plate following rinsing with PBS was counted 24 hours post treatment

Western analysis: Anti-sense transfected cells or control vector transfected cells ($2.5x10^6$ cells/150mm plate) were plated 24 hours prior to treatment with 200 ng/ml anti-Fas antibody (clone CH-11, Kamiya Biomedical Company). 24 hours post treatment cells were washed with PBS and lysed in RIPA buffer (1%Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1mM PMSF, 2mg/ml aprotonin and 2mg/ml pepstatin in PBS). Samples containing 50mg protein were separated by SDS-PAGE and transferred to nitrocellulose membranes. The immunoblots were probed with either anti-Nrf2 antibody (1:100, Santa Cruz, sc722) or anti-bFGF-2 antibody, (1:200, Santa Cruz, sc 079), incubated with goat anti rabbit conjugated to horseradish peroxidase (Pierce) followed by incubation with SuperSignal substrate (Pierce). Following autoradiography, the probes were stripped off (Amersham, ECL Western blotting protocols) and the membranes were hybridized with anti-actin antibody, (1:100, Sigma A4700 or A2066). The intensities of the bands were quantified by the National Institute of Health Image program.

Treatment with N-acetyl cysteine: HeLa cells ($8.3x10^4$ cells /well in 6 wells plates) were plated 20-24 hours prior to treatment with various concentrations of NAC (Sigma/Aldrich,) in the presence or absence of 50 ng/ml anti-Fas antibody (clone CH-11, Kamiya Biomedical Company). The number of viable, trypan blue (Gibco/BRL) excluding cells that remained attached to the plate following rinsing with PBS was counted 5 days post treatment.

Treatment with Dicumarol: HeLa cells, $1.6x10^5$ cells /well were plated in 6 wells plates. 20-24 hours later cells were treated with various concentrations of dicumarol (Sigma/Aldrich,) in 0.2 mM NaOH for 15 minutes prior to the addition of 200 ng/ml anti-Fas antibody (clone CH-11, Kamiya Biomedical Company). The number of viable, trypan blue (Gibco/BRL) excluding cells that remained attached to the plate following rinsing with PBS was counted 17 hours post treatment.

cDNA microarray analysis:. Approximately 500 ng of the PCR products of Pool 1 and Pool 2 (same preparations that were used for the subtraction, before their cleavage by BamHI and HindIII) were labeled with 100 mCi of [^{33}P] dCTP (3000 Ci/mmole, ICN,) by the random primers DNA labeling system (Gibco/BRL), purified (Aershm/Pharmacia, ProbeQuant G50 micro columns) and individually hybridized to Human GeneFilters (GF211, Research Genetics). The filter was pre-hybridized for 40-60 minutes at 68 ^{0}C in ExpressHyb Hybridization solution (Clontech), followed by hybridization for 3-5 hours at 68^{0}C. The filter was washed in 2xSSC, 0.05% SDS at room temperature 3-5 times for 10-15 minutes each time followed by 2 washes for 15 minutes each in 0.1x SSC, 0.1% SDS at 55^{0}C. The image was generated by Molecular Dynamics phospho-imager. In between hybridizations, the probe was stripped off by adding boiling solution of 0.5% SDS and incubating at room temperature for 1 hour. Successful removal of probe was confirmed by phosphor-imager analysis. Images processing and calculation of the ratio of the signals of Pool 2 probe to Pool 1 probe were performed by Pathways II software (Research Genetics). All the spots that showed significant differential abundance were visually inspected. --

Page 51, after "Table 2", please insert the following Table:

--

Gene name	GenBank accession	Fold depletion from

	Number	Pool 2
TNF receptor associated factor 6	AA456295	5.25
Human COP9	AA489699	3.81
Antithrombin III	T62060	3.21
Mucin 1, transmembrane	AA488073	2.81
Casein Kinase, alpha 1	AA625758	2.79
Adenosine receptor A3	AA863086	2.47
calcium/calmodulin-dependent protein kinase II	AA056626	2.42
Human protein immuno-reactive with anti-PTH antibodies	AA088258	2.34
Retinoic acid receptor, gamma 1	AA496438	2.11

--

IN THE CLAIMS:

Please cancel claims 2-19 and insert the following new claims:

2. (New) A method for the identification of genes that encode for inhibitors of cell death comprising the steps of:

inactivating genes in cells by sensitizing cells to cell death, using gene inactivation means;

applying positive selection means to the sensitized cells; and

utilizing subtraction analysis means to identify the genes that have been inactivated.

3. (New) The method according to claim 1, wherein said inactivating step includes transfecting an antisense library in a vector into target cell.

4. (New) A gene identified by the method of claim 1.

5. (New) The gene according to claim 4, wherein said gene is one from the group of genes found in Table 3.

6. (New) The gene according to claim 4, wherein said gene is one from the group consisting essentially of Nrf-2, bFGF.

7. (New) The method according to claim 1, wherein said method is further used for identifying drug interactions.

8. (New) The method according to claim 1, wherein said method is further used for identifying survival factors.

9. (New) The method according to claim 1, wherein said method is further used for identifying targets that inhibit signalling.

Respectfully submitted,

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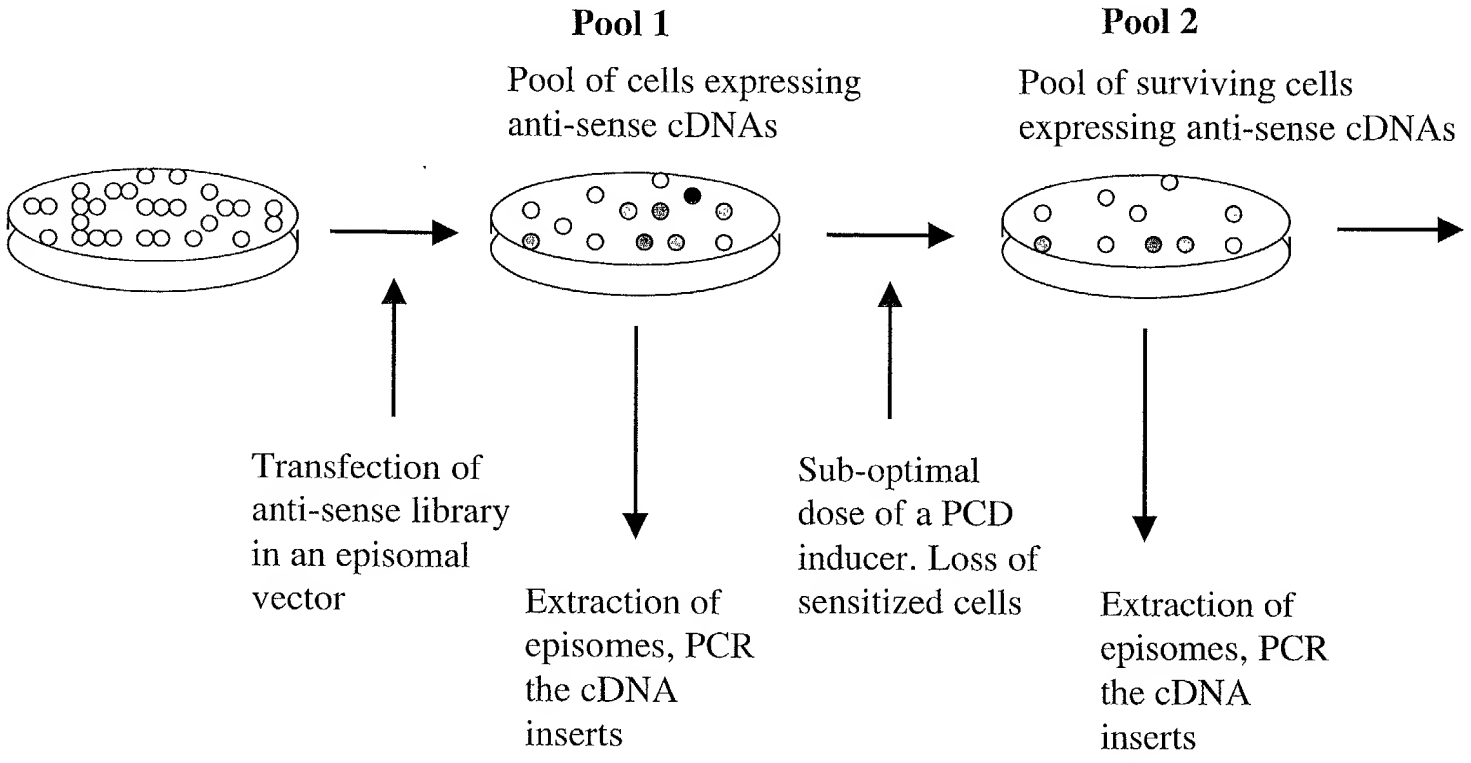
I hereby certify that this paper or fee is being deposited with the United States Postal Service "Express Mail Post Office To Addressee" service under 37 CFR 1.10 on the date indicated above and is addressed to: Assistant Commissioner for Patents, Washington, DC 20231. BOX PATENT APPLICATION.

Constance McLean
Constance McLean

AHM DESIGN



Identification of sensitizing cDNAs that are present in Pool 1 but depleted from Pool 2 by:

1. Subtraction of Pool 2 cDNAs from Pool 1 cDNAs
2. Hybridization of gene array filters with labeled Pool 1 and Pool 2 cDNAs

Fig. 3

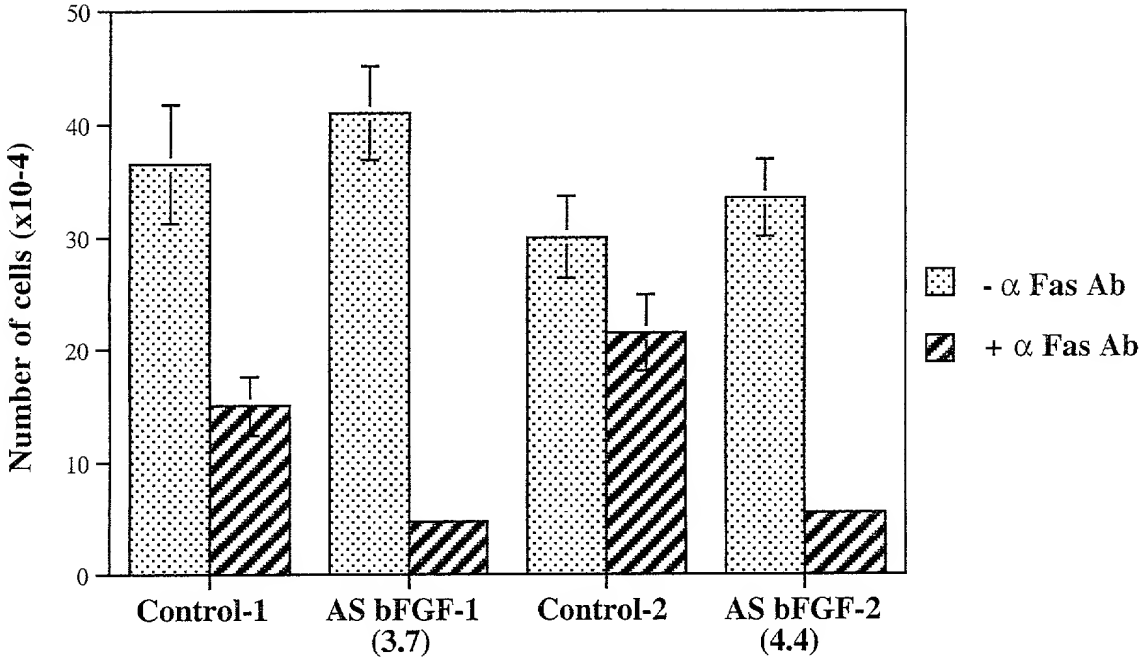

50
40
30
20
10
0
Number of cells (x10-4)
Control-1
AS bFGF-1
(3.7)
Control-2
AS bFGF-2
(4.4)
- α Fas Ab
+ α Fas Ab

FIG. 4A

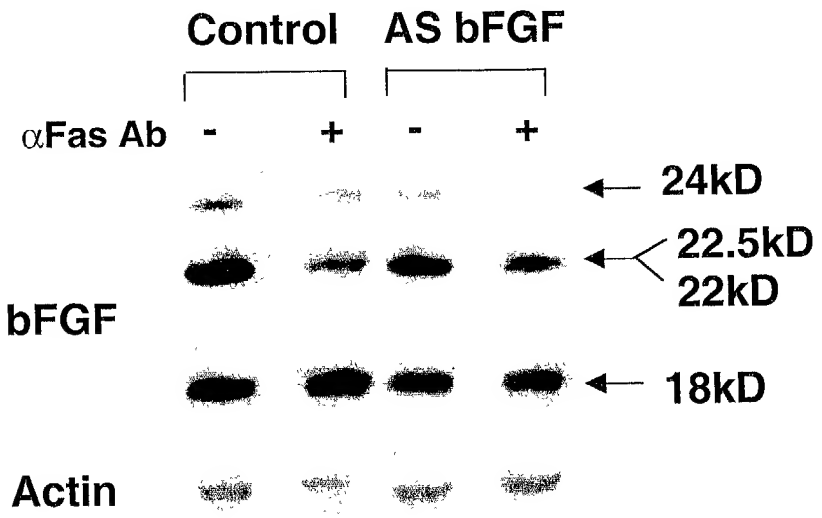


Fig. 4b

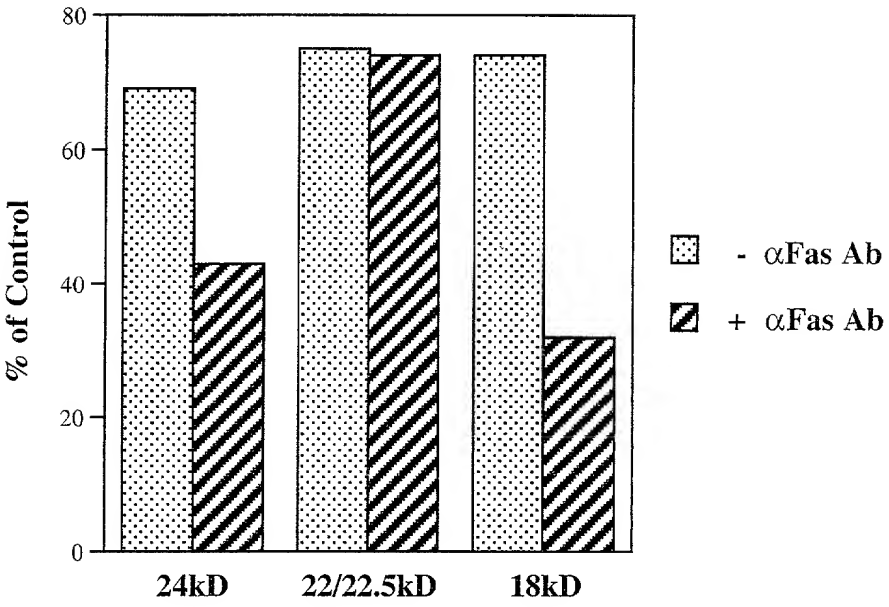


FIG. 4C

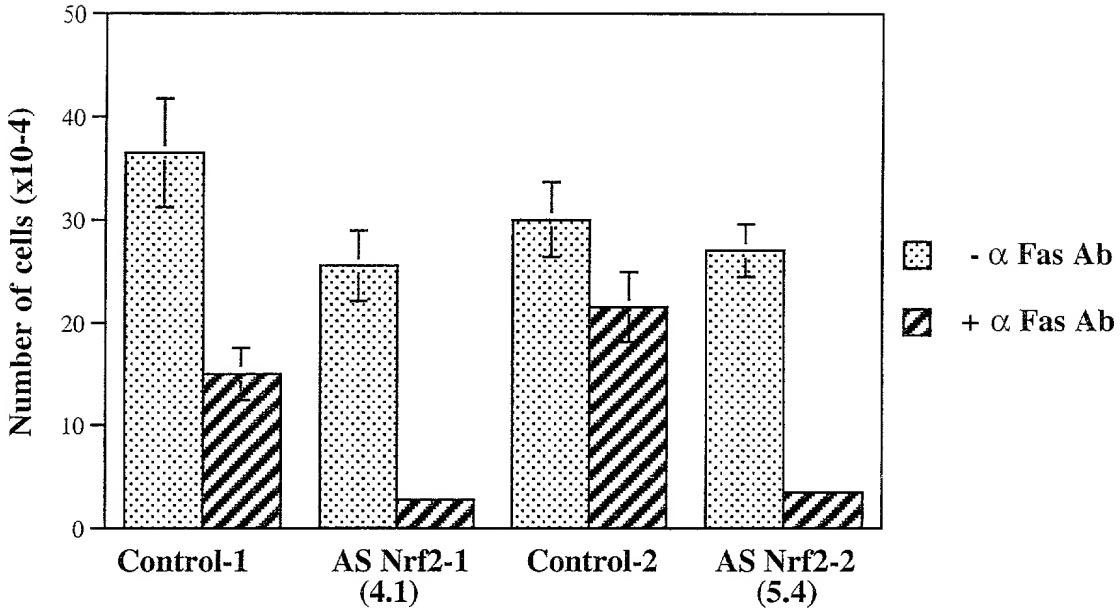

50
40
30
20
10
0
Number of cells (x10-4)
Control-1
AS Nrf2-1
(4.1)
Control-2
AS Nrf2-2
(5.4)
- α Fas Ab
+ α Fas Ab

FIG. 5A

Fig. 5B

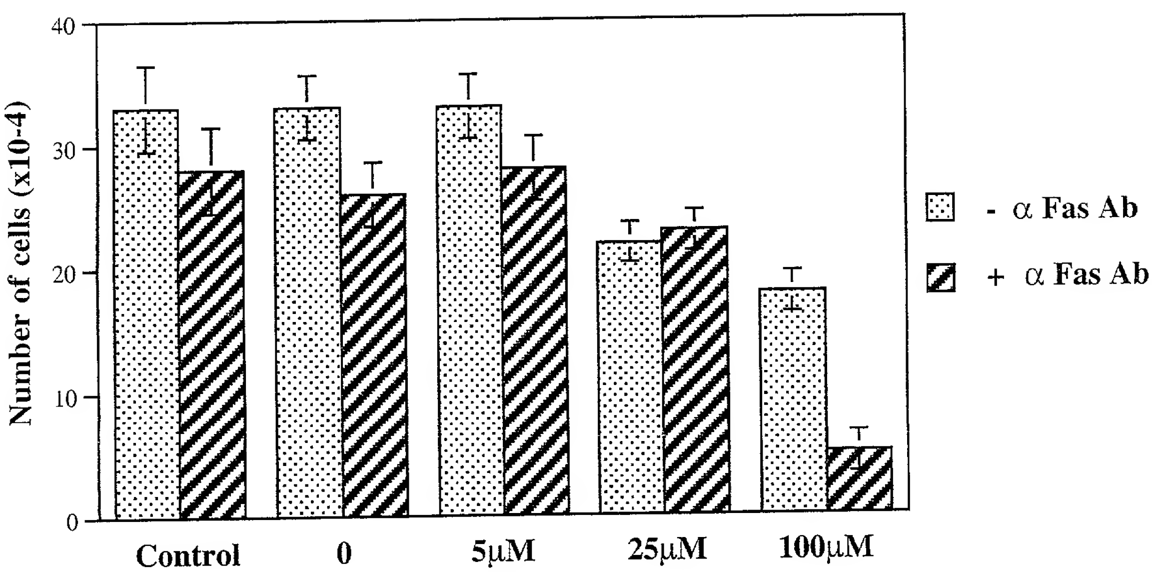

40
30
20
10
0
Number of cells (x10-4)
Control
0
5μM
25μM
100μM
- α Fas Ab
+ α Fas Ab

FIG. 5C

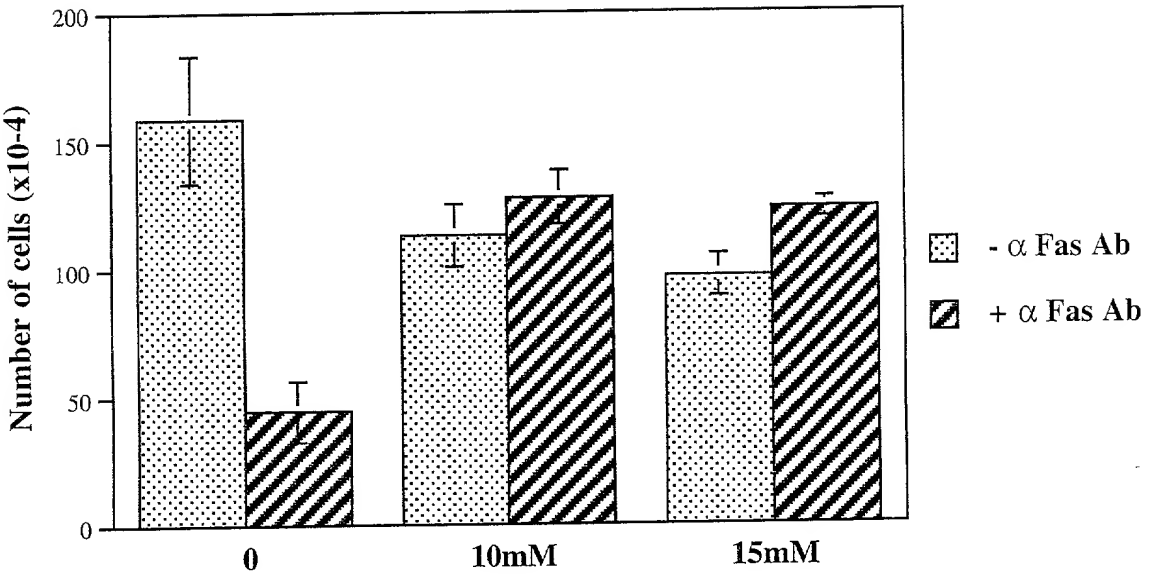

200
150
100
50
0
Number of cells (x10-4)
0
10mM
15mM
- α Fas Ab
+ α Fas Ab

FIG. 5D

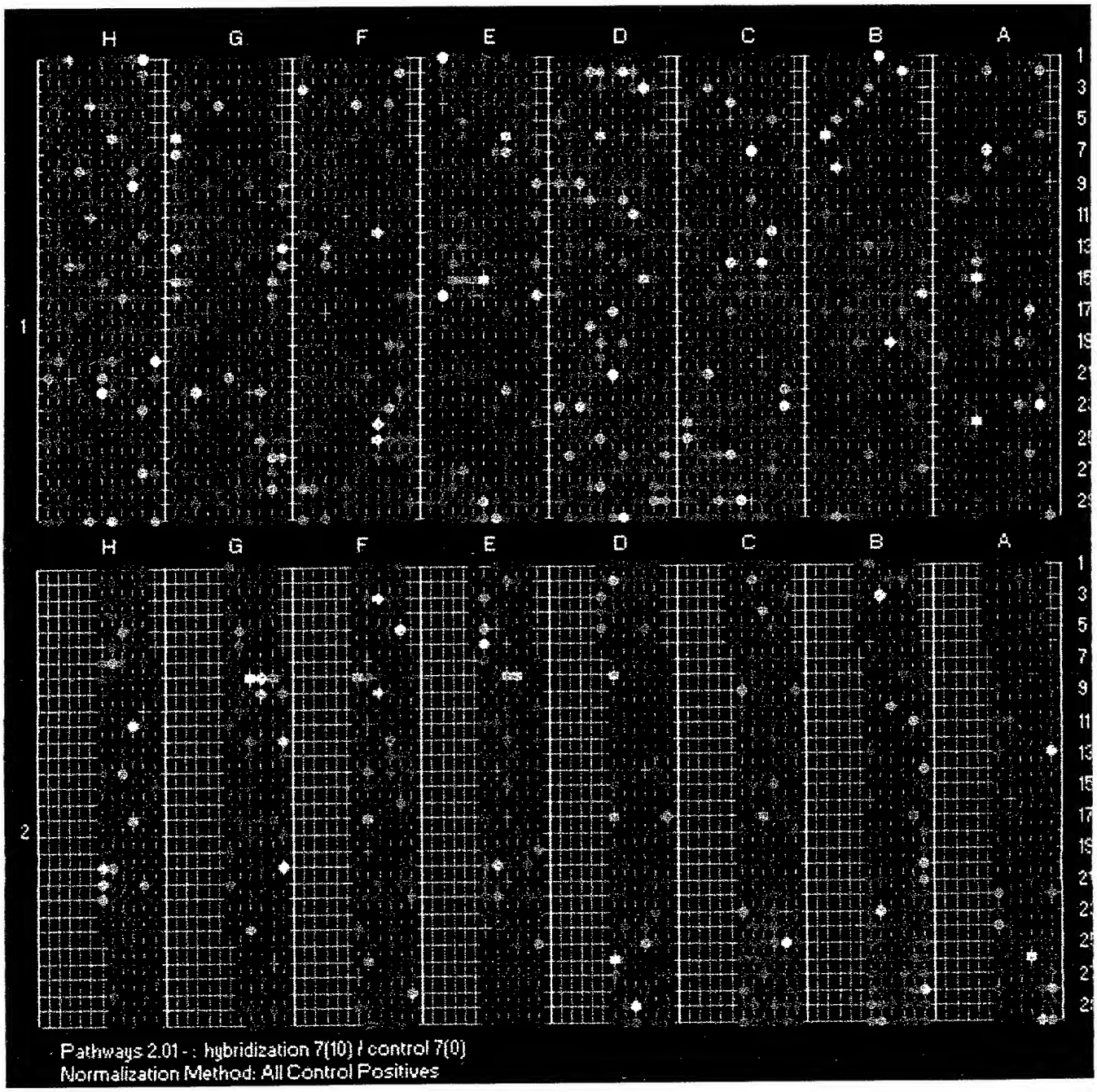

H
G
F
E
D
C
B
A
1
3
5
7
9
11
13
15
17
19
21
23
25
27
29
1
2
Pathways 2.01 - : hybridization 7(10) / control 7(0)
Normalization Method: All Control Positives

FIG. 6A

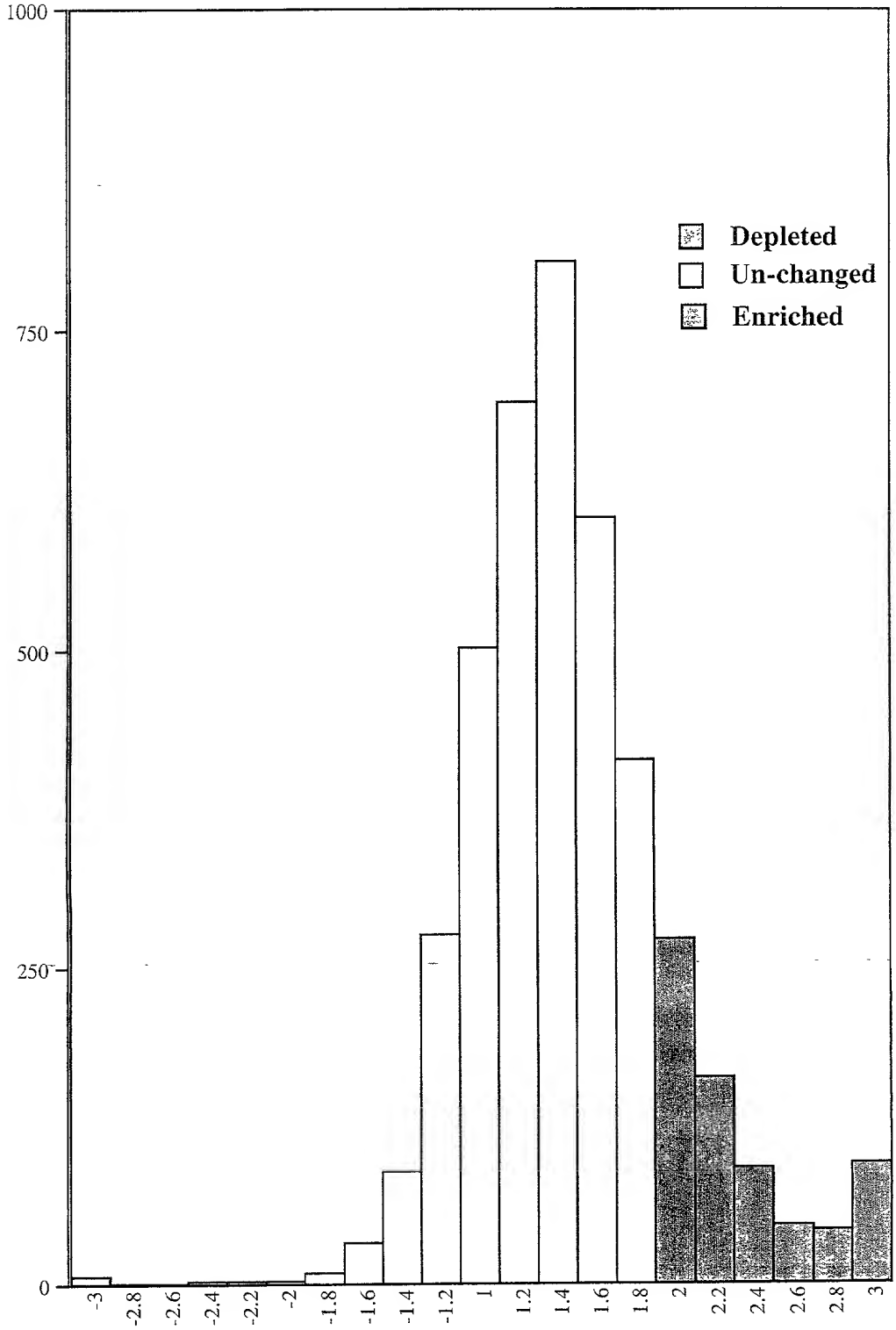

1000
750
500
250
0
Number of clones
Depleted
Un-changed
Enriched
-3
-2.8
-2.6
-2.4
-2.2
-2
-1.8
-1.6
-1.4
-1.2
1
1.2
1.4
1.6
1.8
2
2.2
2.4
2.6
2.8
3
Fold change in abundance in Pool 2 compared to Pool 1

FIG. 6B

Gene name	GenBank accession number	Fold depletion from Pool 2
TNF recpetor associated factor 6	AA456295	5.25
Human COP9 homolog	AA489699	3.81
Antithrombin III	T62060	3.21
Mucin 1, transmembrane	AA488073	2.81
Casein Kinase, alpha 1	AA625758	2.79
Adenosine receptor A3	AA863086	2.47
calcium/calmodulin-dependent protein kinase II	AA056626	2.42
Human protein immuno-reactive with anti-PTH antibodies	AA088258	2.34
Retinic acid receptor, gamma 1	AA496438	2.11

FIG. 6C

Docket No.
0168.00079

Declaration and Power of Attorney For Patent Application

English Language Declaration

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name,

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled

GENE IDENTIFICATION METHOD

the specification of which

(check one)

☐ is attached hereto.

☒ was filed on **February 7, 2000** as United States Application No. or PCT International Application Number _____

and was amended on _____
(if applicable)

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose to the United States Patent and Trademark Office all information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, Section 1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, Section 119(a)-(d) or Section 365(b) of any foreign application(s) for patent or inventor's certificate, or Section 365(a) of any PCT International application which designated at least one country other than the United States, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate or PCT International application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application(s)			Priority Not Claimed
PCT/US97/20989	**PCT**	**12 November 1997**	☐
(Number)	(Country)	(Day/Month/Year Filed)	
			☐
(Number)	(Country)	(Day/Month/Year Filed)	
			☐
(Number)	(Country)	(Day/Month/Year Filed)	

I hereby claim the benefit under 35 U.S.C. Section 119(e) of any United States provisional application(s) listed below:

(Application Serial No.)	(Filing Date)
(Application Serial No.)	(Filing Date)
(Application Serial No.)	(Filing Date)

I hereby claim the benefit under 35 U. S. C. Section 120 of any United States application(s), or Section 365(c) of any PCT International application designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of 35 U.S.C. Section 112, I acknowledge the duty to disclose to the United States Patent and Trademark Office all information known to me to be material to patentability as defined in Title 37, C. F. R., Section 1.56 which became available between the filing date of the prior application and the national or PCT International filing date of this application:

09/284,782	**6 July 1999**	**Allowed**
(Application Serial No.)	(Filing Date)	(Status) (patented, pending, abandoned)
(Application Serial No.)	(Filing Date)	(Status) (patented, pending, abandoned)
(Application Serial No.)	(Filing Date)	(Status) (patented, pending, abandoned)

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

POWER OF ATTORNEY: As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith. *(list name and registration number)*

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Full name of sixth inventor, if any
Sixth inventor's signature Date
Residence
Citizenship
Post Office Address

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